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(54) Title: NOVEL METHOD OF LARGE SCALE PLASMID PURIFICATION

(57) Abstract

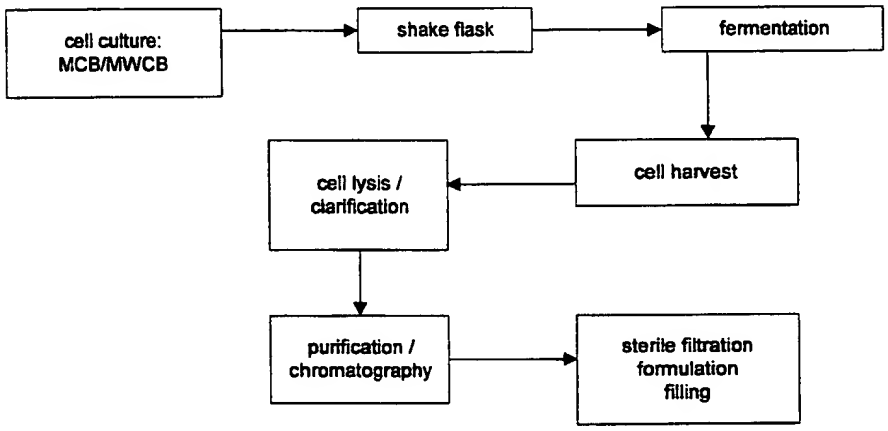
A method of purifying pharmaceutical grade plasmid DNA from host cells is disclosed. The method involves a single, "mixed mode" anion exchange step which employs a stringent ethanol wash to remove endotoxins and other impurities from more hydrophilic plasmid DNA.

NOVEL METHOD OF LARGE SCALE PLASMID PURIFICATION

Background of The Invention

With the increasing value of recombinant DNA in therapeutic applications, the need for methods for obtaining sufficient amounts of highly pure plasmid DNA from cell cultures has grown.

Typically, the manufacture of large scale plasmid DNA involves the following steps.



Within this general scheme, chromatographic purification of DNA presents perhaps the biggest hurdle based on the physical characteristics of the biomolecule as well as the intrinsic impurities derived from the host cell, for example, *E. coli.* The chief impurities which hinder the purification of plasmid DNA are the large amounts of polymers of similar structure (chromosomal DNA and RNA) and high levels of endotoxin.

Plasmid DNA is a highly anionic polymer which is sensitive to shear and to degradation by nucleases. Plasmids are as large or larger than the pores of almost all chromatographic media. Several chromatographic procedures for the purification of biologically active plasmid DNA (without the use of CsCl - ethidium bromide ultracentrifugation) have been developed, at least at laboratory scale, including gel filtration chromatography, hydroxyapatite chromatography, acridine yellow affinity

while they enhance the resolution and separation of the "mini prep" anion exchange matrices, they are totally unsuitable for use within a regulatory compliant cGMP production process.

Improved methods for obtaining large scale plasmid DNA in a highly pure form suitable for therapeutic use would be highly beneficial.

Summary of the Invention

The present invention provides a fully scaleable method for isolating plasmid DNA to a level of purity which meets regulatory standards required for the manufacture of recombinant protein pharmaceuticals. The method provides the advantage of streamlining the purification process so that a single anion exchange step is capable of producing pharmaceutical grade plasmid DNA. The method provides the further advantage of not requiring the use of enzymes or other agents capable of damaging the plasmid DNA, such as lysozyme, RNase, Proteinase K, phenol, chloroform, and ethidium bromide.

In one embodiment, the method of the invention calls for lysing host cells and obtaining a lysate; applying the lysate to an anion exchange column; washing the column with a solution comprising a sufficient amount of ethanol to substantially remove endotoxin bound to the column, without substantially removing plasmid DNA bound to the column; eluting plasmid DNA bound to the column; and collecting chromatographic fractions of the elute. The elute can then be assayed for purity using, for example, standard protein, DNA and/or endotoxin assays. Plasmid DNA present in the elute can be assayed for retained function using, for example, expression assays.

Further steps can optionally be performed in the method of the invention. In one embodiment, the lysate is clarified by filtering to remove large cellular debris prior to applying the lysate to the anion exchange resin. The lysate can also be concentrated to remove RNA precipitate. In a preferred embodiment, the lysate is mixed with a non-ionic detergent, such as 2% triton X-114, prior to applying the lysate to the column. Preferably, the detergent is one which flows through the anion exchange column and does not interfere with plasmid DNA binding to the resin. By performing this initial

Figure 5 is a chromatogram showing plasmid purification and endotoxin removal by 2% triton X-114 phase partitioning, followed by TMAE chromatography using a 40% ethanol + 5% acetic acid wash step.

Figure 6 1% shows PAGE (1% agarose gel) analysis of plasmid purification using TMAE standard chromatography.

Figure 7 shows PAGE (1% agarose gel) analysis of plasmid purification using 2% triton X-114 phase partitioning, followed by TMAE chromatography.

Figure 8 shows PAGE (1% agarose gel) analysis of plasmid purification using 2% triton X-114 phase partitioning, followed by TMAE chromatography using a 40% ethanol + 5% acetic acid wash step.

Figure 9 shows graphic and tabular results from transfection expression assays using plasmid DNA encoding α-interferon, purified by the method of the invention.

Detailed Description of the Invention

The present invention provides a novel "mixed" mode plasmid purification method which relies on differences in both charge and hydrophilicity between plasmid DNA and cellular impurities, such as endotoxins and other protein contaminants, to isolate plasmid DNA. These differences are exploited, in a single chromatography step, to produce plasmid DNA to a level of purity which meets regulatory standards required for the manufacture of recombinant protein pharmaceuticals (e.g., < 0.1 EU/μg DNA). The chromatography step employs both anion exchange and charge separation strategies, using a stringent ethanol wash, to separate endotoxins from plasmid DNA.

As in all plasmid purification protocols, the method of the invention initially calls for lysis and plasmid extraction from host cells containing the desired (e.g., recombinant) plasmid. This can be done, for example, by adding cells to alkaline buffers, such as 0.2 NaOH/1.5% SDS, as is well known in the art. Preferably the lysis

the final purification of plasmid DNA. These chromatography techniques are well known in the art and can be practiced as described, for example, in US Patent No. 5,561,064, the contents of which are incorporated by reference herein.

In the present invention, anion exchange chromatography is performed to separate plasmid DNA molecules from contaminating molecules based on molecular ionic charge or isoelectric point (pI) at a given pH. Ion exchange columns may be packed with positively charged beads (for anion exchangers) that make up the support matrix. The charge density and pI of the molecules will determine the ionic capacity of the support matrix that is suitable for separating the molecules. Ion exchange operations may be run using two different mobile phases or buffers (i.e., under gradient conditions). The starting buffer may be a low salt or ionic concentration buffer. The eluting buffer may have a significantly higher ionic concentration than the starting buffer. The operating pH can be determined by sample solubility and support matrix stability. For example, an ion exchanger may be run at a pH of about 6-11 and a linear gradient developed between about 0.3 and 1.0M NaCl. Water miscible organic solvents (for example, acetonitrile) may be used to decrease retention time, but it is preferred that the use of organic solvents be avoided so that the storage of organic chemical waste is precluded.

In one embodiment, the invention employs a trimethylaminoethyl (TMAE) fractogel anion exchange resin. The resin is packed into a standard, preferably large-scale column, such as a Pharmacia XK 50 column, at a suitable bed height (e.g., approximately 20.5 cm) and a suitable total column volume (e.g., approximately 400 ml). The column is then run on a suitable preparative HPLC at a linear flow rate of e.g., 150 cm/hr. Chromatographic profiles are then monitored at two different wavelengths, for example, 260 nm and 280 nm, and peak fractions collected based on their real time chromatographic profiles.

Following packing, the column is equilibrated and the sample containing the plasmid DNA is loaded onto the column. Typically, the first wash contains relatively high salt (e.g., 0.68 M NaCl) and results in selective binding of components contained in the sample to the column, and removal of most of the detergent. This optional wash is

convention of performing hydrophilic chromatography at a low pH. The lower pH of the wash step can also aid in residual RNA removal.

A second column step also may be incorporated within the production process to add a degree of ruggedness and to ensure rigorous product control.

Following collection of elute, samples can be tested for plasmid DNA purity, quality and quantity using a number of assays known in the art. For example, to assay for endotoxin levels, a Limulus Amboycte Lysate Assay (LAL) kinetic assay can be performed on individual plasmid fractions according to manufacturer protocols. To analyze purified plasmid DNA for total size, and for the presence of residual genomic DNA and RNA, standard polyacrylamide gel (e.g., 1%) electrophoresis (PAGE) can be used. To analyze plasmid DNA concentration, individual fractions can be analyzed by optical density (OD) at a wavelength of 260 nm using and extinction coefficient of 50 μg / ml. To analyze samples for residual protein content, a Micro Bicinchoninic Acid (BCA) Assay kit (e.g., Pierce Micro BCA) can be used according to manufacturer protocols.

In addition and, typically, as a final step, purified plasmid DNA samples can be assayed for activity using expression or other functional assays. Transfection efficiency of plasmid DNA can be used, for example, using reporter genes or genes encoding protein which is then quantified in standard immunoassays.

The invention shall be further illustrated in the following examples which are not intended to be limiting.

EXAMPLES

EXPERIMENTAL MATERIALS

"TEG" Buffer (25 mM Tris / 10mM EDTA / 50mM Glucose, pH 7.5)

0.2 NaOH + 1.5% SDS Buffer

3 M Potassium Acetate + 5 M Acetic Acid Buffer, pH 5.6 (KAc / AA)

"TE" Buffer (50mM Tris +10mM EDTA, pH 7.5)

Ammonium Acetate (NH_4Ac)

$CoCl_2 \cdot 6H_2O$, 0.12 g/L

HCl, 37 ml

Glucose 4 g/L

Fermentation Media

K_2HPO_4, 8.25 g/l

$(NH_4)_2SO_4$, 5.625 g/L

$(NH_4)_2HPO_4$, 8.25 g/L

$MgSO_4 \cdot 7H_2$, 0.5 g/L

Yeast Extract, 10 g/L

$FeCl_3 \cdot 6H_2O$, 0.2025 g/L

$CaCl_2 \cdot 2H_2O$, 0.1875 g/L

$ZnSo_4 \cdot 7H_2O$, 0.877 g/L

$MnCl_2 \cdot 4H_2O$, 0.0375 g/L

$CuSO_4 \cdot 5H_2O$, 0.0094 g/L

H_3BO_3, 0.00375 g/L

$(HN_4)_6Mo_7O_{2/1} \cdot 4H_2O$, 0.00375 g/L

$CoCl_2 \cdot 6H_2O$, 0.00375 g/L

Antifoam, 0.05 ml/L

Thiamine, 0.31 g/L

L-leucine, 0.31 g/L

Feed Medium

Glucose, 640 g/L

Yeast Extract, 100 g/L

$MgSO_4 \cdot 7H_20$, 12 g/L

Beckman J2-MI Centrifuge

Komposite 6 x 1 L Rotor

Materials and Equipment necessary to perform 3-QC-0048 (Kinetic LAL Assay)

grown to an optimal density for transfer to the fermenter as determined by optical density (OD) at 600nm.

The Fed-Batch Fermentations were run in a B.Braun Biostat C-30 Fermenter with a starting working volume of 24L. The basal medium was inoculated with a 10% inoculum and fed using a mathematical model. The feed rate was adjusted as growth slowed to maintain less than 1.0 g/L glucose concentration in the fermenter.

Feed Equation:

$F = FO^* \exp(\mu(t-t_0))$, where:

$F0 = (\mu^*X)/(Y^*S)$

F, F0 in ml/hr (rate)

μ in h-1 (specific growth rate)

t in hours of fermentation

X in g total initial biomass

Y in g/g (yield of cell dry wt per gram of glucose)

S in g/ml (concentration of feed)

During fermentation, the aeration was maintained at 1vvm (vol. of air per vol. of liquid per minute) and dissolved oxygen was controlled at 20% by agitation. The pH was maintained at 7.0 with 28-30% NH_4OH. Temperature was held constant at 32°C in order to maintain a maximum growth rate (μ) of 0.31 Upon inoculation, the "feed" medium (see Appendix 1) was fed into the fermenter according to the feed rate profile. The duration of the fermentations were ~22 hrs or until late log phase was reached. Cells were then harvested by centrifugation and frozen at -20°C.

CELL LYSIS AND PLASMID EXTRACTION

To 2000g of cells (wet weight), 9.0 L of TEG buffer was added until the cells were completely thawed and dispersed in solution. To this cell suspension, 21.0 L of 0.2 N NaOH +1.5 % SDS buffer was added and incubated at room temperature with thorough mixing for twenty minutes. After twenty minutes, 16.5 L of KAc / AA buffer

which the effects of column capacity were further addressed. Prior to loading the column, all plasmid samples were adjusted to a conductivity of between 38 mS -42 mS and adjusted to a pH of 7.5 The column was run on a PerSeptive BioSystems BioCAD 250 preparative HPLC at a linear flow rate of 150 cm/hr. Chromatographic profiles were monitored at two wavelengths: 260 nm and 280 nm. All peak fractions were manually collected based on their real time chromatographic profiles.

The chromatographic protocol, optimized for the removal of genomic DNA and residual RNA, is briefly summarized as follows:

Column Equilibration:	50 mM Tris, pH 7.5, 500 mM NaCl
Sample Load:	Plasmid in 50 mM Tris, pH 7.5, 500 mM NaCl
Column Wash:	50 mN Tris, pH 7.5, 0.68 M NaCl
Stringent Wash:	**40 % Ethanol + 5 % Acetic Acid**
Column Elution:	50 mM Tris, pH 7.5, 1.2 M NaCl
Column Cleaning:	0.2 N NaOH + 1 M NaCl
Column Regeneration:	0.1 N HCl + 1 M NaCl

LIMULUS AMBOYCTE LYSATE ASSAY

The LAL kinetic assay (to assay for levels of endotoxin) was performed on individual plasmid fractions according to SOP 3-QC-0048

QUANTITATION AND ANALYSIS OF PLASMID DNA

Plasmid DNA was analyzed for total size and for the presence of residual genomic DNA and RNA by 1 % agarose gel electrophoresis using the Uni-Lane™ Gel System (0.5 x TBE 1 % GT-1) from Geno Technology, Inc., St. Louis, MO. The molecular weight marker was the 10 kb UniMarker™ from Geno Technology.

Plasmid DNA concentrations for individual fractions were determined at a wavelength of 260 nm using and extinction coefficient of 50 µg / ml.

its binding capacity for plasmid DNA. Even though column capacity experiments had shown no loss of resolution or yield at concentrations of 7.5 mg plasmid / ml resin, endotoxin levels at this loading concentration were unacceptably high. Small scale anion exchange purification can reduce endotoxin levels to acceptable levels but large columns, used at their maximum capacity, do not have sufficient endotoxin clearance. As shown in Table 2, the current industry specification for endotoxin in parenteral gene therapy DNA formulations is

< 0.1 EU / μg DNA (VICAL inc., Qiagen GmbH) .

TABLE 1: CHROMATOGRAPHY RESULTS

Expt. Condition	Total DNA	Resin volume (ml)	Load (mg /ml)	Total Endotoxin Load (EU)	EU / ml of resin	DNA Yield (mg)	EU/μg of DNA
Standard	3000	400	7.5	2.4×10^7	6.0×10^4	368	1.633
Standard	310	100	3.1	6.8×10^5	6.8×10^3	20.8	.046
Standard + Ethanol Wash	310	100	3.1	6.8×10^5	6.8×10^3	16.5	Unde-tectable
Standard + Ethanol Wash	1242	400	3.1	1.0×10^7	2.5×10^4	235	0.112
Standard + Triton	1242	400	3.1	1.0×10^7	2.5×10^4	191	0.016
Standard + Triton + Ethanol	1242	400	3.1	1.0×10^7	2.5×10^4	208	0.014

greater than 10 fold reduction in final endotoxin levels was achieved. While these experiments were carried out using endotoxin free buffers, they were not performed in a purification suite (class 1000 or better). The chromatographic profile (Figure 4) remains similar to the standard chromatographic method. It was, however, found to be necessary to lower the wash step following the ethanol + acetic acid wash to 0.5 M NaCl (rather than a continuation of the 0.68 M NaCl wash step) in order not to lose product.

To streamline the large scale process and eliminate the need for subsequent centrifugation steps, a 2 % Triton X-114 phase partitioning step was used to remove endotoxin prior to anion exchange chromatography. Triton X-114, being a nonionic detergent, flows through the column and does not interfere with plasmid binding to the resin. The chromatographic profile and 1 % agarose gel form this method can be seen in Figure 3 and Figure 7, respectively. What is apparent is that there is some loss of product during the 0.68 M NaCl wash step (lane 8 on the agarose gel) and a slightly lower column yield (see table 1). What is not as easily quantified is the residual amount of Triton X-114 present in the final plasmid product. During the chromatography procedure, it was possible to visualize Triton in all the wash fractions (a phase separation could also be induced) and in the earliest eluting plasmid fraction, and it was observed that the presence of Triton X-114 interfered with the LAL kinetic assay.

Accordingly, as part of the invention, both the 2 % Triton X-114 pre-chromatography step and the stringent ethanol wash step were combined into the chromatography process. The results can be seen in Figure 5 and Figure 8. By including the ethanol wash step, the lowest endotoxin levels were achieved in the final plasmid product as well as no visible loss of product during the 0.68 M NaCl wash step. Equally significant, the 40 % ethanol +5 % acetic acid wash step stripped off the residual Triton X-114. The most likely explanation for Triton X-114 being present is that it must remain associated with biomolecules bound to the column. The ethanol wash step (and subsequent salt wash) offers a major improvement over using just 2 % Triton as the sole endotoxin removal method.

DNA. The plasmid DNA from this large scale process is capable of transfection efficiencies equal to laboratory standards.

Equivalents

Although the invention has been described with reference to its preferred embodiments, other embodiments can achieve the same results. Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific embodiments described herein. Such equivalents are considered to be within the scope of this invention and are encompassed by the following claims. All publications cited herein, including patents and pending patent applications, are hereby incorporated by reference in their entirety.

9. The method of claim 7, further comprising removing the detergent phase formed after adding the non-ionic detergent to the cell lysate, prior to applying the cell lysate to the column.

10. The method of claim 1, further comprising collecting the eluted plasmid from the column.

11. The method of claim 1, further comprising washing the column with a salt solution prior to washing the column with the ethanol solution.

12. The method of claim 1, further comprising:

clarifying the cell lysate to remove large cellular debris; and

concentrating the cell lysate to remove RNA precipitate, prior to applying the lysate to the column.

13. A method of purifying plasmid DNA from a cell lysate comprising:

applying a cell lysate to an anion exchange column;

washing the column with a solution comprising a sufficient amount of ethanol to substantially remove endotoxin bound to the column, without substantially removing plasmid DNA bound to the column;

eluting plasmid DNA bound to the column.

14. The method of claim 13, further comprising adding a non-ionic detergent to the cell lysate prior to applying the lysate to the column.

15. The method of claim 14, wherein the detergent is Triton X-114.

16. The method of claim 13, wherein the solution for washing the column comprises at least 40% ethanol.

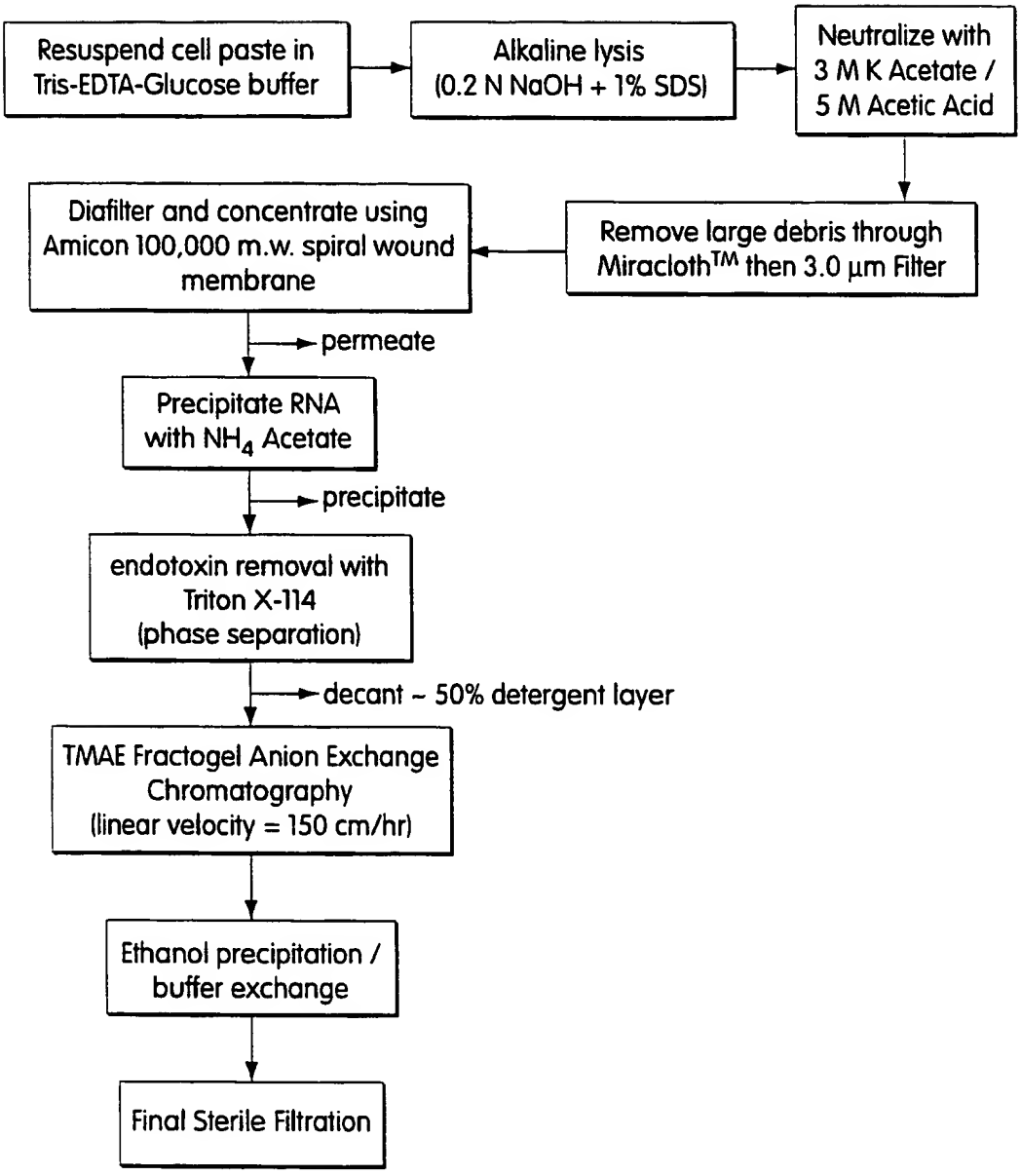

Resuspend cell paste in Tris-EDTA-Glucose buffer
Alkaline lysis (0.2 N NaOH + 1% SDS)
Neutralize with 3 M K Acetate / 5 M Acetic Acid
Remove large debris through Miracloth™ then 3.0 µm Filter
Diafilter and concentrate using Amicon 100,000 m.w. spiral wound membrane
permeate
Precipitate RNA with NH_4 Acetate
precipitate
endotoxin removal with Triton X-114 (phase separation)
decant ~ 50% detergent layer
TMAE Fractogel Anion Exchange Chromatography (linear velocity = 150 cm/hr)
Ethanol precipitation / buffer exchange
Final Sterile Filtration

Fig. 1

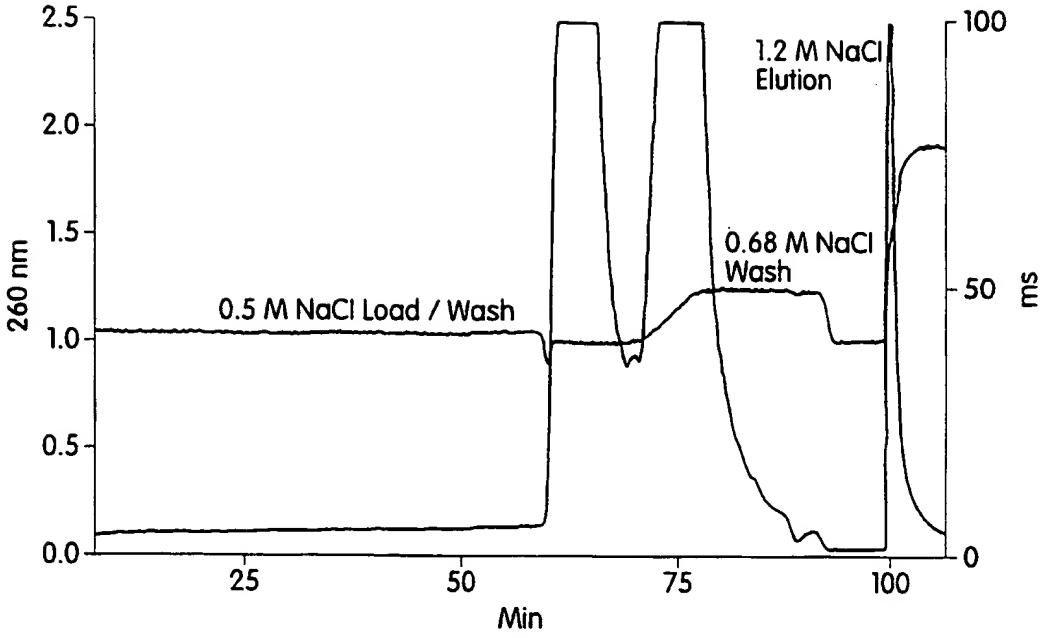

2.5
2.0
1.5
1.0
0.5
0.0
260 nm
25
50
75
100
Min
100
50
0
ms
1.2 M NaCl
Elution
0.68 M NaCl
Wash
0.5 M NaCl Load / Wash

Fig. 3

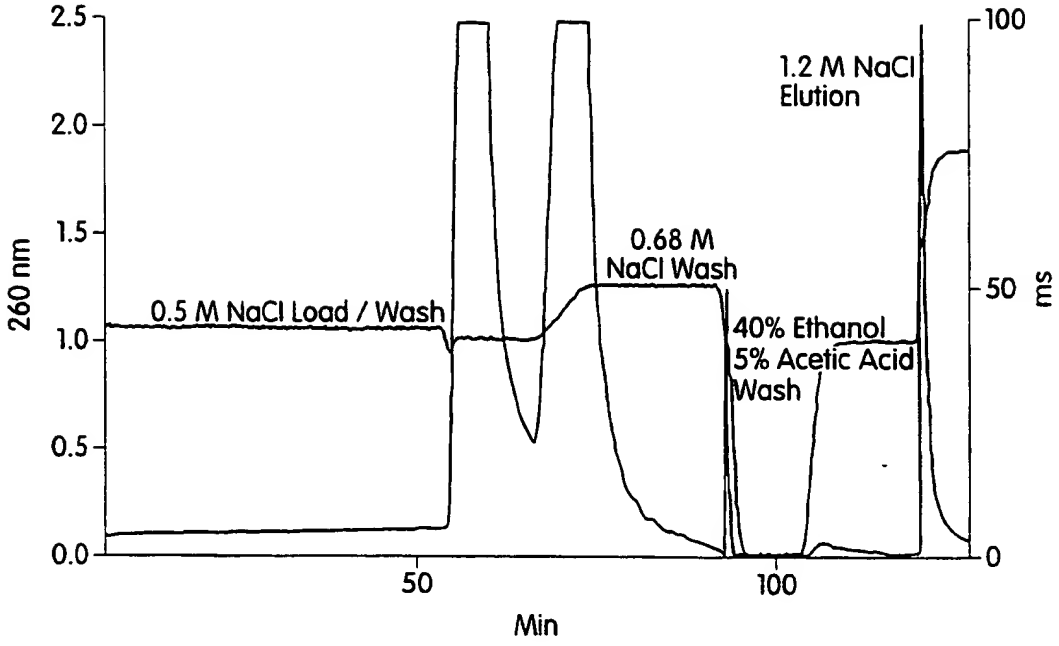

2.5
2.0
1.5
1.0
0.5
0.0
260 nm
0.5 M NaCl Load / Wash
0.68 M
NaCl Wash
40% Ethanol
5% Acetic Acid
Wash
1.2 M NaCl
Elution
100
50
0
ms
50
100
Min

Fig. 5

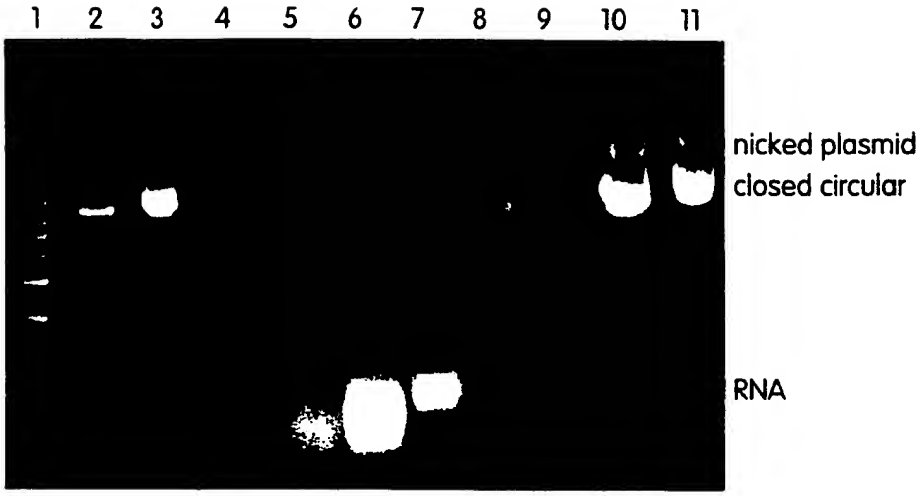


Fig. 7

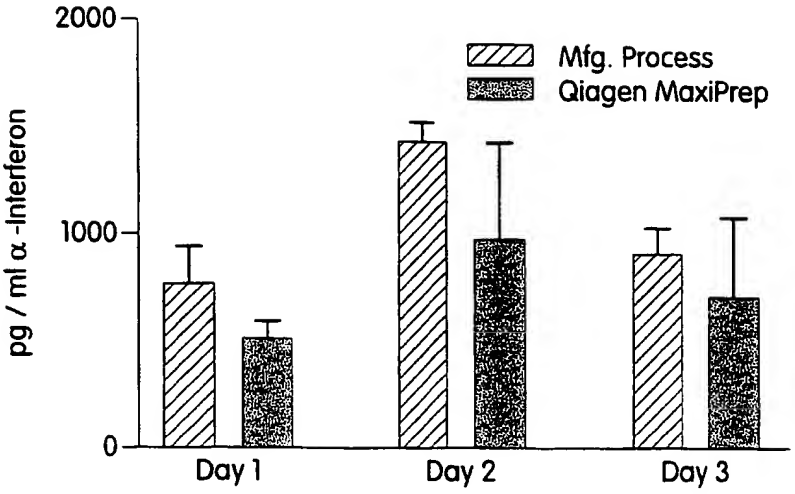

2000
1000
0
pg / ml α -Interferon
Mfg. Process
Qiagen MaxiPrep
Day 1
Day 2
Day 3

Fig. 9

INTERNATIONAL SEARCH REPORT

Inter tional Application No
PC1/US 99/11588

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	SCHORR J ET AL: "LARGE SCALE PURIFICATION OF ENDOTOXIN-FREE PLASMID DNA FOR GENE THERAPY RESEARCH" GENE THERAPY, vol. 1, no. SUPPL. 02, 18 November 1994 (1994-11-18), page S07 XP000572703 ISSN: 0969-7128 abstract	1,2
A	SAMBROOK J: "MOLECULAR CLONING, PASSAGE" MOLECULAR CLONING, LABORATORY MANUAL, vol. 1, 1989, page 1.34/1.35 1.35 XP002011439 SAMBROOK J;FRITSCH E F; MANIATIS T the whole document	1,7-9, 14,15
A	HINES R N ET AL: "LARGE-SCALE PURIFICATION OF PLASMID DNA BY ANION-EXCHANGE HIGH- PERFORMANCE LIQUID CHROMATOGRAPHY" BIOTECHNIQUES, vol. 12, no. 3, 1 March 1992 (1992-03-01), pages 430-434, XP002012269 ISSN: 0736-6205 the whole document	1,2,7,9, 10,12,14

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